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SUBJECT OF INVESTIGATION

CORRELATION BETWEEN SUSCEPTIBILITY TO ORAL INFECTION AND INTESTINAL BACTERIAL FLORA IN THE INBRED MOUSE STRAINS RAISED BY SELECTIVE BROTHER-SISTER MATINGS IN OUR LABORATORY

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SCORPE OF SERVICES

1) <u>Mass breed of two inbred mouse strains</u>.

As regards the strain dd/Ks, the mass breeding has been progressed successfully. The rate of conception has increased gradually, and the average of the rate in past 3 months has reached 61.0 %. The size of litter was 6.0 in average. The total output of mouse in past 3 months was 1,242 mice and monthly output in average came to 410 mice. As this number of mouse was in excess of the expected production, sufficient number of mouse was provided for experimental use and this condition of animal supply would be expected in future.

As regards the strain dd/Kf, massproduction was failed through unknown reason. Though one substrain of dd/Kf has been isolated, which has the ability of 3 conceptions, by developing its subline as much as possible, almost all the mice produced has to be used for examination of their susceptibility to the infection and for keeping the strain, so no mice were provided for research work.

2) <u>Study of the relation between normal intestinal flora and the infection</u>.

<u>a</u>. <u>Significance of Enterococci as normal intestinal flora to the infection</u>.

(1) Evaluation of the effect of antibiotics as pretreatment-agent.

(a) The effect of neomycin in vitro. From several past experiences it was known that the number of intestinal flora in mice decreased markedly when 2,000 γ of streptomycin and 1,000 γ of erythromycin were administered simultaneously to commercial mice orally. But in the case of dd/Ks strain mice relatively large number of the bacteria remained regardless of the administration of these antibiotics. To overcome this phenomenon, several new antibiotics were examined insted of streptomycin. As a criterion for selection of new antibiotics, their character of slow or non-absorption from the intestines was seriously considered. Because it was easily expected that if the drug absorbed so much in the body, it might be soon excreted again to the intestines and the following infection might be influenced by it.

From these view point, neomycin and colimycin were chosen and their bacteriostatic effect were tested in vitro against the bacteria isolated from feces of mice. The result was shown in table 1. Neomycin was more effective than colimycin, and the effect of combined administration of both drugs was not superior than the effect of single use of neomycin.

(b) Experiments in vivo. In this experiment 32 mice were divided into 4 droups (A, B, C, and D). 24 hours after oral administration of 10,000 γ of neomycin through metal tube, 0.1 mg of Enterococcus viridans, which had been isolated from feces of mouse, was administered orally to group A mice, once daily for three successive days. 24 hours after the last administration of the drug, moderate number of Salmonella enteritidis strain No. 11, which has high virulence for mice, was given to the mice orally.

Group B mice were administered orally 10,000 γ of neomycin and 3 days later Salmonella enteritidis Strain No. 11 was given to them.

Group C mice were challenged with Salmonella enteritidis strain No. 11, just 24 hours after the administration of neomycin.

Group D was control group, and challenged with strain No. 11 without any pretreatment.

These 4 groups were observed for 3 weeks after the challenge, but it was difficult to find any difference between the results of 4 groups. (Fig. 1)

From these results neomycin was regarded to be unsuitable for the present purpose.

(c) The effect of colimycin in vivo. As stated above, bacteriostatic action of colimycin in vitro was inferior to that of neomycin. But when sufficient dose of colimycin was given to mice, the number of gram negative bacilli in fecal culture decreased markedly. From these results, it was decided to adopt the combined pretreatment with colimycin and erythromycin in further experiments.

(2) Evaluation of the antagonistic effect of Enterococcus viridans to Salmonella infection.

(a) Experimental infection with Salmonella enteritidis. 50 mice were provided for this experiment and they were divided into 4 groups.

Group A mice were given 200,000 units of colimycin and 2,000 γ of erythromycin. After that, 1 mg of Enterococcus was given to them orally in three successive days. Then, 680 of living cells of Salmonella enteritidis was inoculated orally.

Group B was treated just the same as group A except the change of bacterial species which was used in pre-infection. That is, 1 mg of E. coli, which was isolated from normal mouse, was given orally insted of Enterococcus.

Group C was given the same antibiotics and 24 hours after the treatment, the same strain of Salmonella was inoculated.

Group D mice were served for control, the same number of the same strain of Salmonella as used in the other groups was given to them without any pretreatment. The results were shown in fig. 2.

In group A, 8 out of 10 mice survived and infected Salmonella was proved from blood of only 2 mice died. These results were similar to that of group D.

In group B, half of the animals survived and 2 of them became carrier. Other half of animals died from Salmonella infection.

In group C, only 1 out of 10 mice survived and other 9 mice died from Salmonella infection.

From these results it was presumed that the susceptibility of dd/Ks mice to Salmonella infection was raised by the administration of antibiotics, but it decreased to the normal level when enterococcus existed in the intestines and the same effect as Enterococcus was shown by E. coli, even it was not so high.

(b) Rise and fall in number of bacterial flora following Salmonella infection. For this experiment 84 mice were provided and they were divided into 4 groups. Each group was treated just in the same way as described in (2) - (a). Three mice from each group were sacrificed and served for each days experiment. That is, they were sacrificed on the days, before and after pretreatment, after preinfection, and 4 successive days following challenge with Salmonella. Upper and lower parts of the intestines were cut out in 3 cm lengths and cultured on SS agar, Drigalski BTB agar, 10 % horse blood agar, Staphylococcus medium No. 110 and Rogosa medium respectively. After 24 hours cultivation, every colony, which looks different under operating microskope by means of oblique illumination, was isolated and morphologically classified into 4 groups ---- gram positive bacillus, gram positive coccus, gram negative bacillus and others.

The members of gram negative bacillus were cultured on SIM, TSI and glucose phosphate pepton solution, and fermentation of lactose, glucose and sucrose, production of H_2S and indol, motility and PPA and VP reaction were tested. The member of gram positive cocci were examined on their hemolysis, heat-resistance, growth in pH 9.6 broth, broth containing 6.5 % NaCl and bile broth, and also examined the precipitation test with anti-group D immune serum, if necessary. In the case of culture on Rogosa medium, the culture was incubated for 72 hours and bacteria grown on them were examined morphologically.

In these examinations the number of Salmonella, E. coli, Streptococcus group, Staphylococcus, Lactobacillus group were counted respectively on SS agar, BTB agar, blood agar, Staphylococcus medium No. 110, and Rogosa medium. In figs. 3, 4, 5, and 6, the number of flora in upper part of the intestines was shown. As challenged Salmonella could not be detected on SS agar and the number of bacteria grown on Rogosa medium was so stable, these data were excluded from the figures.

Enterococcus persisted markedly for a long time after the inoculation though least differences were obserbed between each mice.

Streptococcus disappeared completely after the pretreatment with antibiotics. Though in groups B, C, and D Streptococcus reappeared on the next day of pretreatment, in group A the appearance was postponed 24 more hours, possibly because of the influence of heavy growth of Enterococcus.

Staphylococcus was not influenced by pretreatment and there was no significant changes throughout the experiment.

The most members of E. coli group disappeared after the pretreatment but reappeared so easily. From some mice in group B, any member of E. coli group was recovered but in these mice Proteus group was isolated in large number. In general, Proteus did not appeared in any regularity.

(c) Influence of the pretreatment and preinfection to invasiveness of Salmonella. 72 mice were treated and infected in the same way to that of (2) - (a). Each group cocsisted of 18 mice, and 3 mice of each group sucrificed in successive 6 days after the challenge with Salmonella, and heart blood, the liver spleen, kidney, mesenterial lymphgland, taken from them, were cultivated in selenite broth and on BTB agar. The results were shown in table 2.

In group A, there were no mice which were proved the invasion of Salmonella into organ, except one mouse which harbored the Salmonella in the livor and spleen. In group B, there were 3 mice which were proved the Salmonella in their body. In group D, there was only one mouse from which the Salmonella was isolated. In group C, on the contrarly, the invasion of the Salmonella was proved in all mice, since on the 2nd day of challenge.

b. Significance of E. coli as normal intestinal flora to the infection. As described in (2) - (a), one strain of E. coli, isolated from normal mouse, was administered orally and examined the antagonistic effect to Salmonella infection. But the effect was lower than that of Enterococcus. This result was not so difficult to understand theoretically. Essentially, E. coli will grow at lower part of the intestines, while the invasion of Salmonella will happen at upper part of the intestines. So the antagonism between them will not occur directly. In this meaning the antagonism might be expected to occur between E. coli and Shigella, when the later grows at lower part of the intestines of mice.

3) Examine genetical characteristics of each inbred mouse strain by reciprocal matings and cellular and humoral factors making up the host defence to infection of mice.

When Salmonella inoculated into peritoneal cavity of mouse immunized with killed vaccine, the number of Salmonella decreased markedly and disappeared in short period. These clearance ability can be transferred passively between the same species by transferring peritoneal cell suspensions of immunized animals.

But when the cells were killed before transfer, the clearance does not occur. So the destiny of the transferred cells in recipient can be presumed from the existence of clearance ability. These technics were used in this project to examine genetical characteristics of dd/Ks strain mouse. The method used was as follows: glycogen-induced peritoneal cells (mostly monocytes) were collected from mice previously immunized with heat-killed vaccine. After being washed twice, monocytes were transferred to normal mice which were then challenged intraperitoneally with Salmonella. In this experimental condition, when the challenge with Salmonella was carried out in commercial adult mouse within 6 days after the transfer, the clearance ability remains, but when the challenge was delayed more than 8 days from the day of transfer, the clearance ability decreased markedly (Fig. 7). This phenomenon might indicate that the transferred cells were rejected through the realization of homotransplantation immunity, and that the commercial mice lack genetical homogenity.

On the other hand, in case of adult mice of dd/Ks strain the clearance was recognized markedly even after 110 days after transfer, and from this fact the genetical homogenity in dd/Ks strain was proved. (Fig. 8)

Examination by the method of reciprocal matings has not been carried out.

TAB I. EFFECT OF NEOMYCIN AND COLIMYCIN IN VITRO

NO. OF SAMPLE	NEOMYCIN			COLIMYCIN			NM. + COM.			
	50 γ	5	0.5	2.5*	0.25	0.02	25 + 1.2	2.0 + 0.1	02 + 0.0	0.02 + 0.001
1	−	+	++	−	+	+	−	+	+	++
2	−	+	++	+	+	+	−	+	+	++
3	−	+	+	+	+	+	−	+	+	+
4	−	+	+	+	+	+	−	+	+	++
5	−	+	+	+	+	+	−	+	+	+
6	−	+	+	−	+	+	−	+	+	+
7	−	+	+	−	+	+	−	+	+	+
8	−	+	+	+	+	+	−	+	+	+
9	−	+	++	−	+	+	−	+	+	++
10	−	+	++	+	+	+	−	+	+	+
11	−	+	++	−	+	+	−	+	+	+
12	−	+	+	−	+	+	−	+	+	+
13	−	+	+	+	+	+	−	+	+	+
14	−	+	++	+	+	+	−	+	+	+
15	−	+	+	−	+	+	−	+	+	+
16	−	+	+	+	+	+	−	+	+	+
17	−	+	++	−	+	+	−	+	+	++
18	−	+	++	+	+	+	−	+	+	++
19	−	+	+	−	+	+	−	+	+	+
20	−	+	+	−	−	−	−	+	+	+

* MILLION UNIT / ML

TAB.2. INFLUENCE OF THE PRETREATMENT AND PREINFECTION TO INVASIVENESS OF SALMONELLA ENTERITIDIS IN MICE.

GROUP \ ORGAN \ DAY	1					2					3					4					5					6				
	H	L	S	K	M	H	L	S	K	M	H	L	S	K	M	H	L	S	K	M	H	L	S	K	M	H	L	S	K	M
A	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−
B	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−
	−	−	−	−	−	−	−	−	−	−	−	+	−	−	+	−	−	−	−	+	+	+	+	+	+	−	−	−	−	−
C	−	−	−	−	−	−	+	−	−	+	+	+	+	−	−	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−
	−	−	−	−	−	−	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	−	−	−	−	−	−	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

H: HEART BLOOD, L: LIVER, S: SPLEEN, K: KIDNEY,
M: MESENTERIAL LYMPHGLAND.

FIG. 1. THE EFFECT OF NEOMYCIN FOR PRETREATMENT

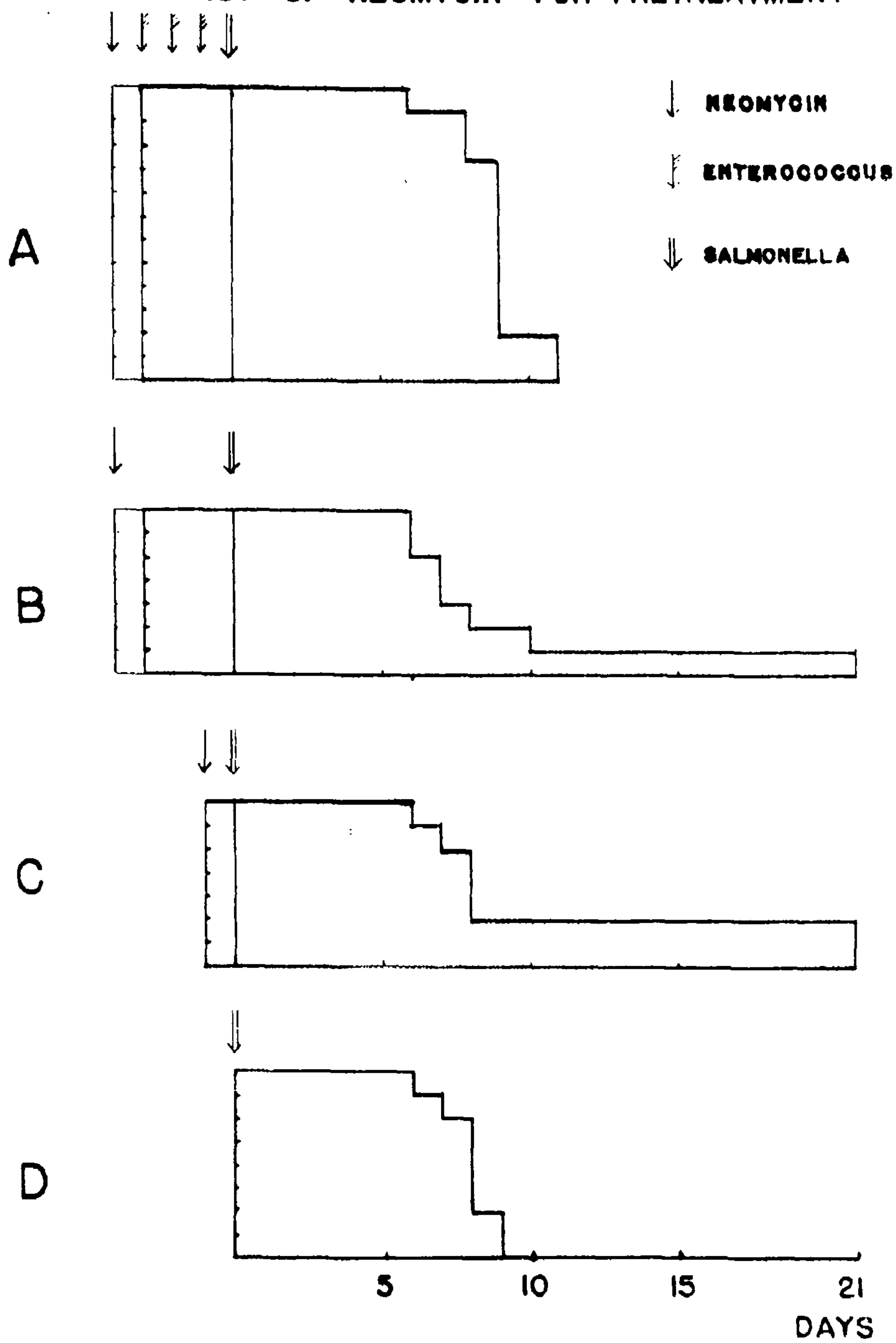

FIG. 2. EVALUATION OF THE ANTAGONISTIC EFFECT OF ENTEROCOCCUS VIRIDANS TO SALMONELLA INFCTION

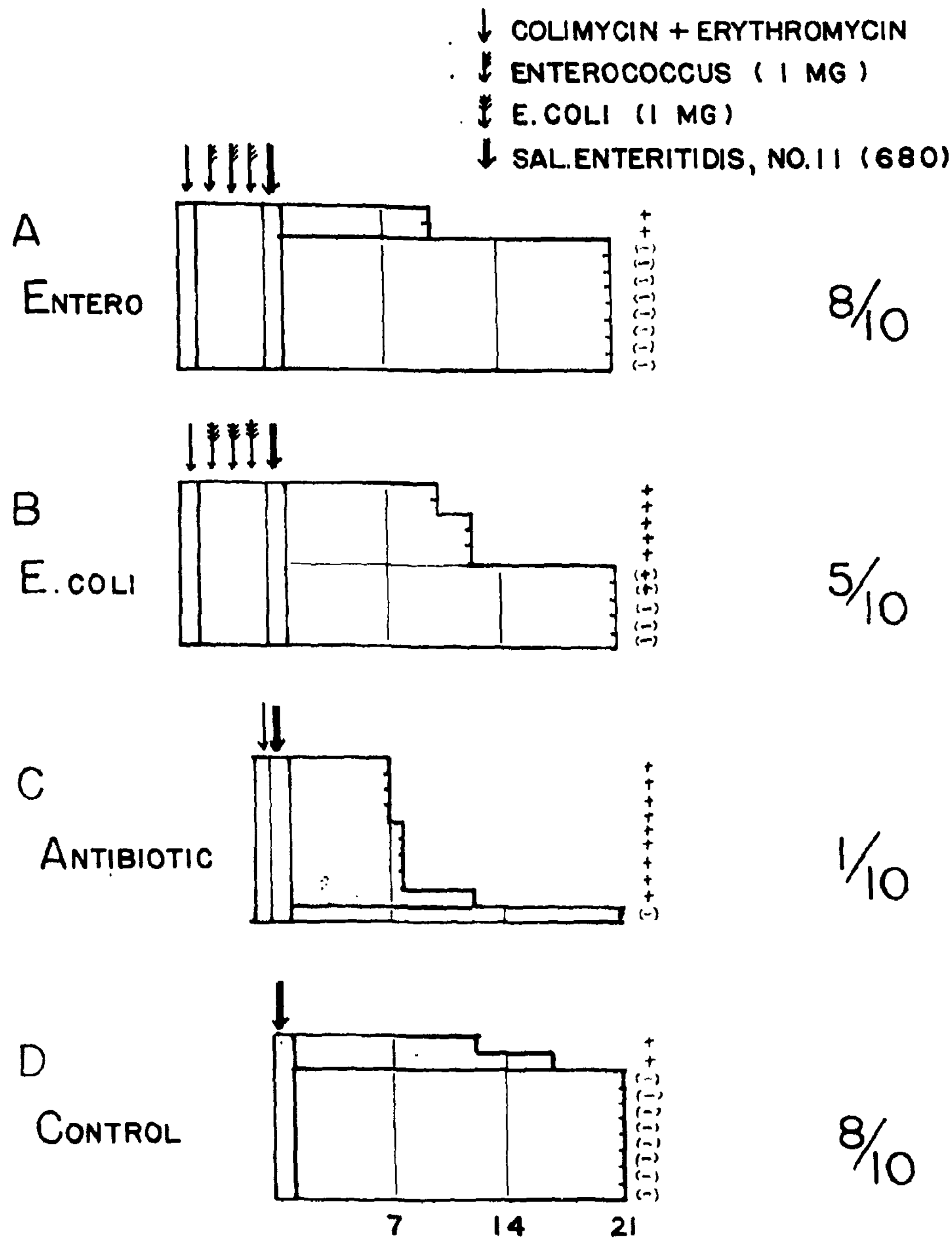

FIG. 3. RISE AND FALL OF THE NUMBER OF INTESTINAL BACTERIA (GROUP A)

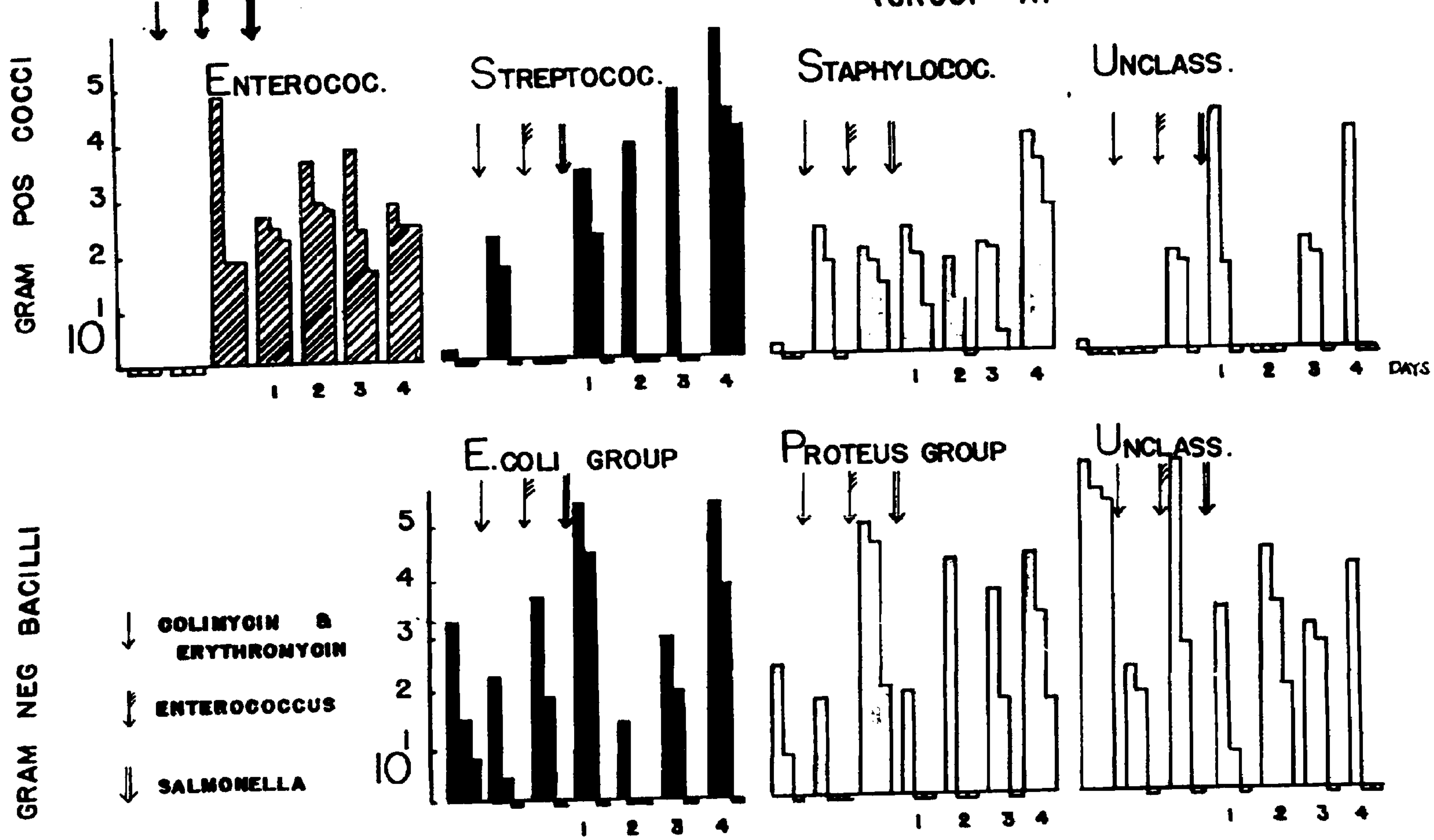

FIG. 4.

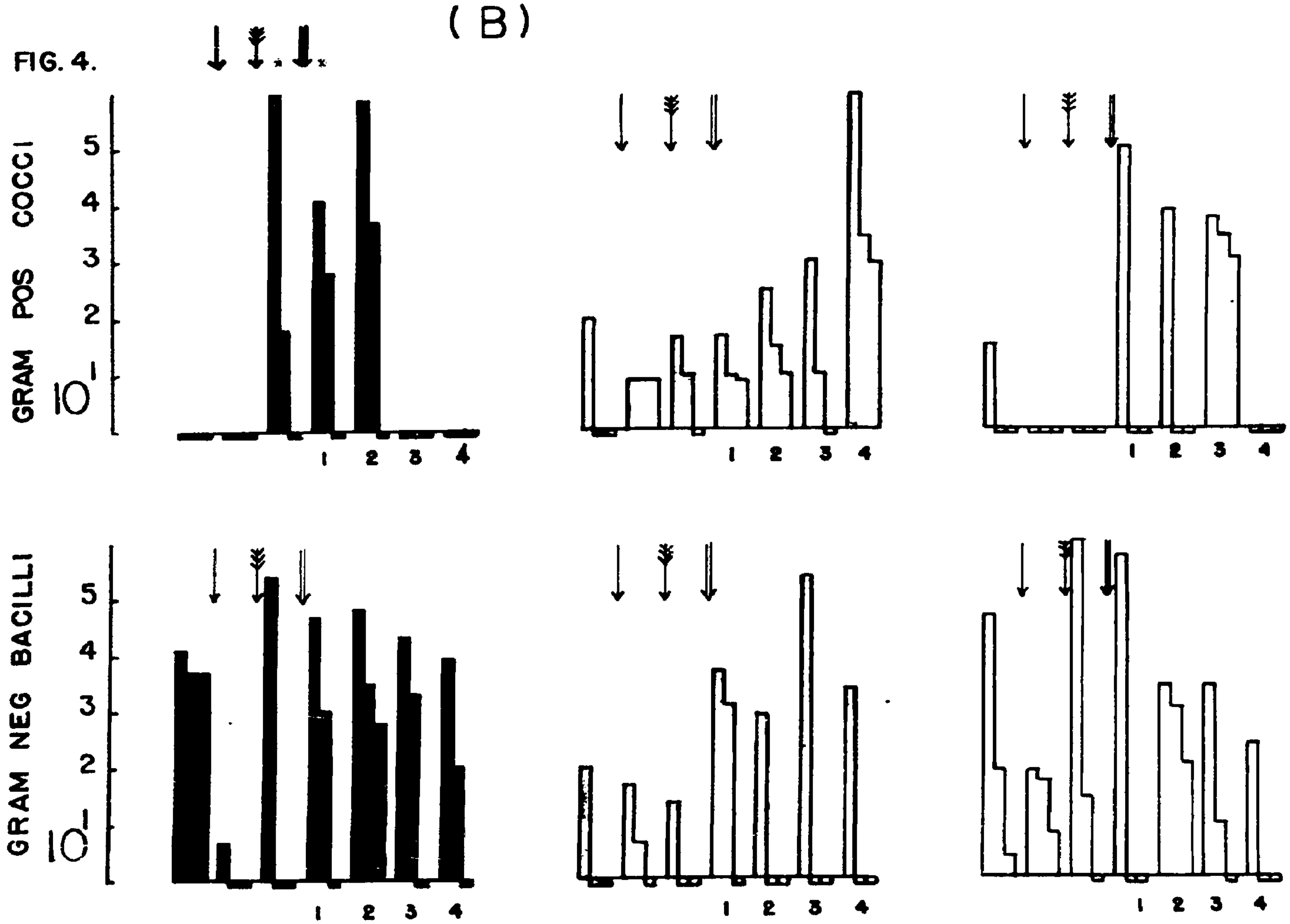

FIG. 5.

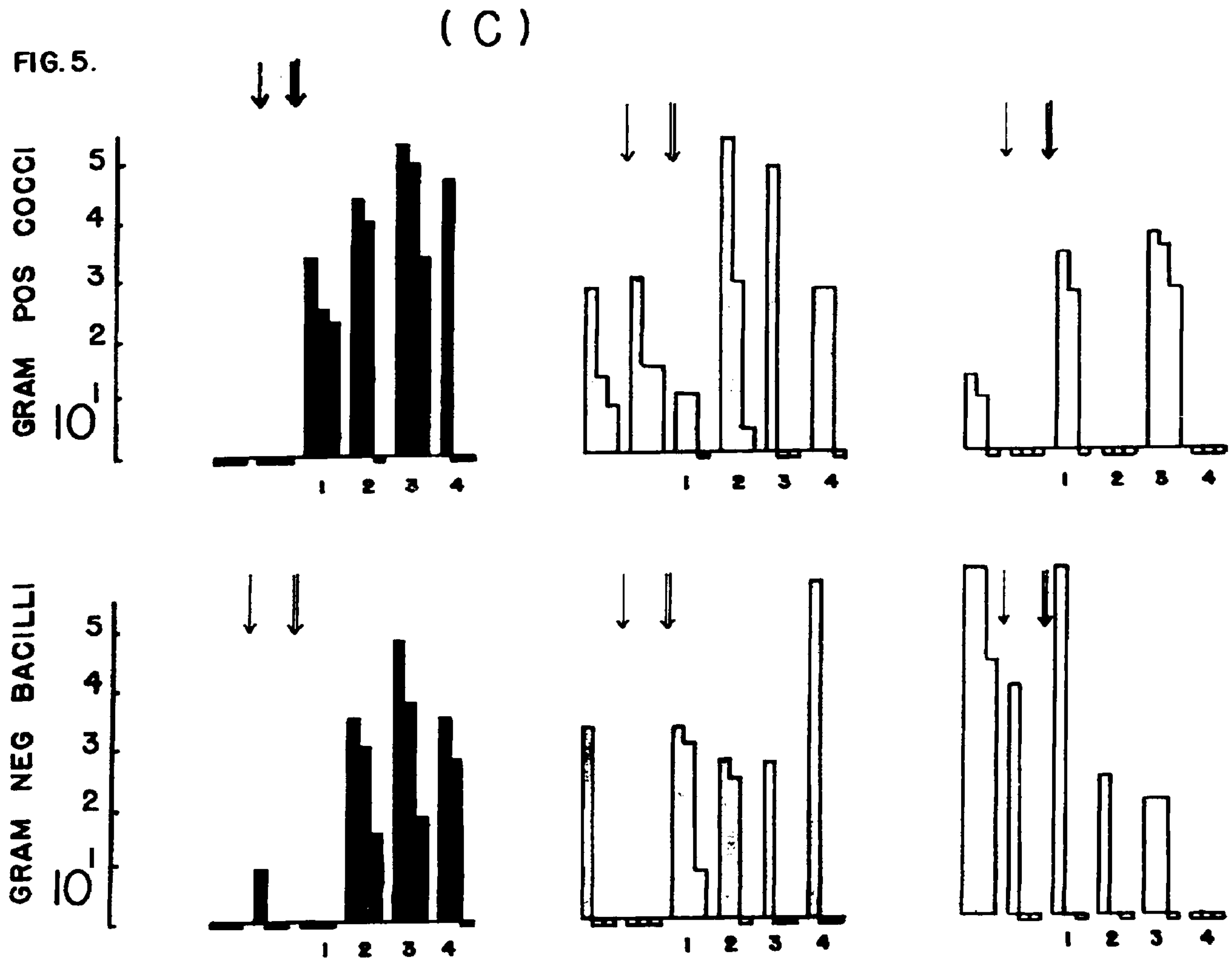

FIG. 6.

(D)

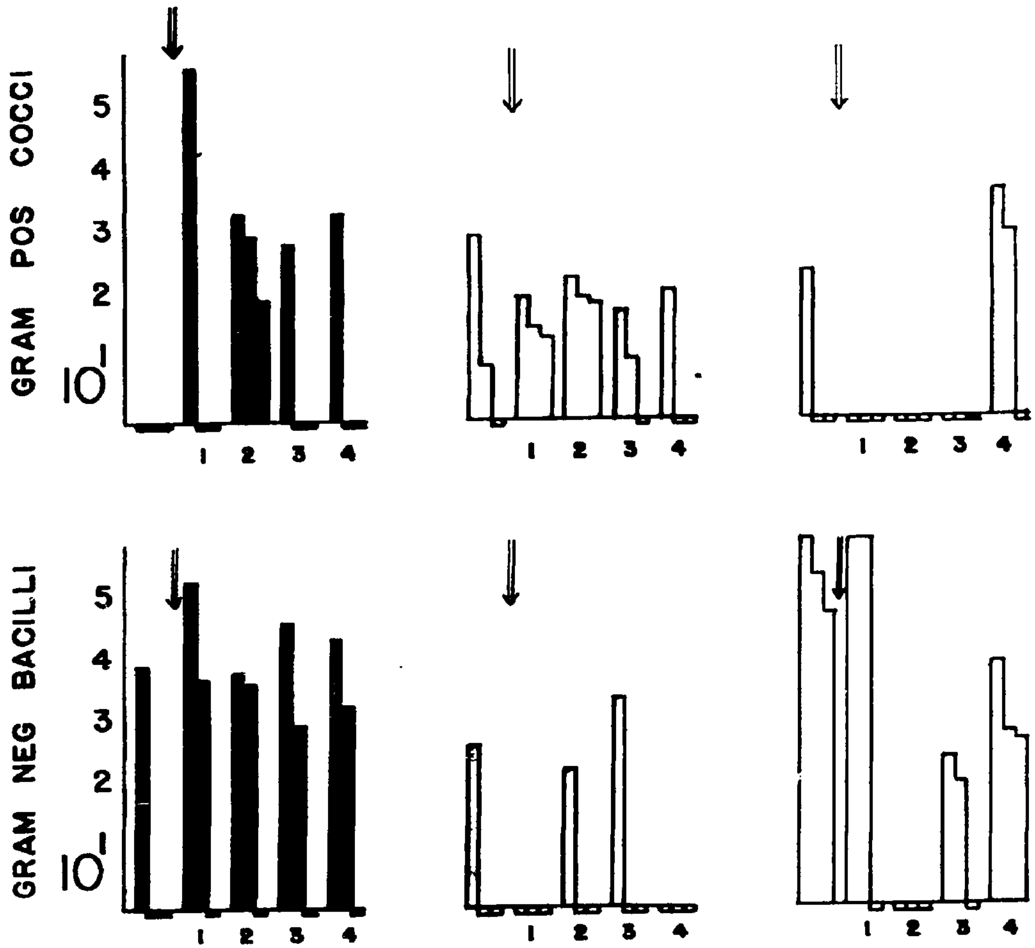

FIG. 7. INFLUENCE OF TIME FACTOR TO CLEARANCE EFFECT

(COMMERCIAL ADULT MOUSE)

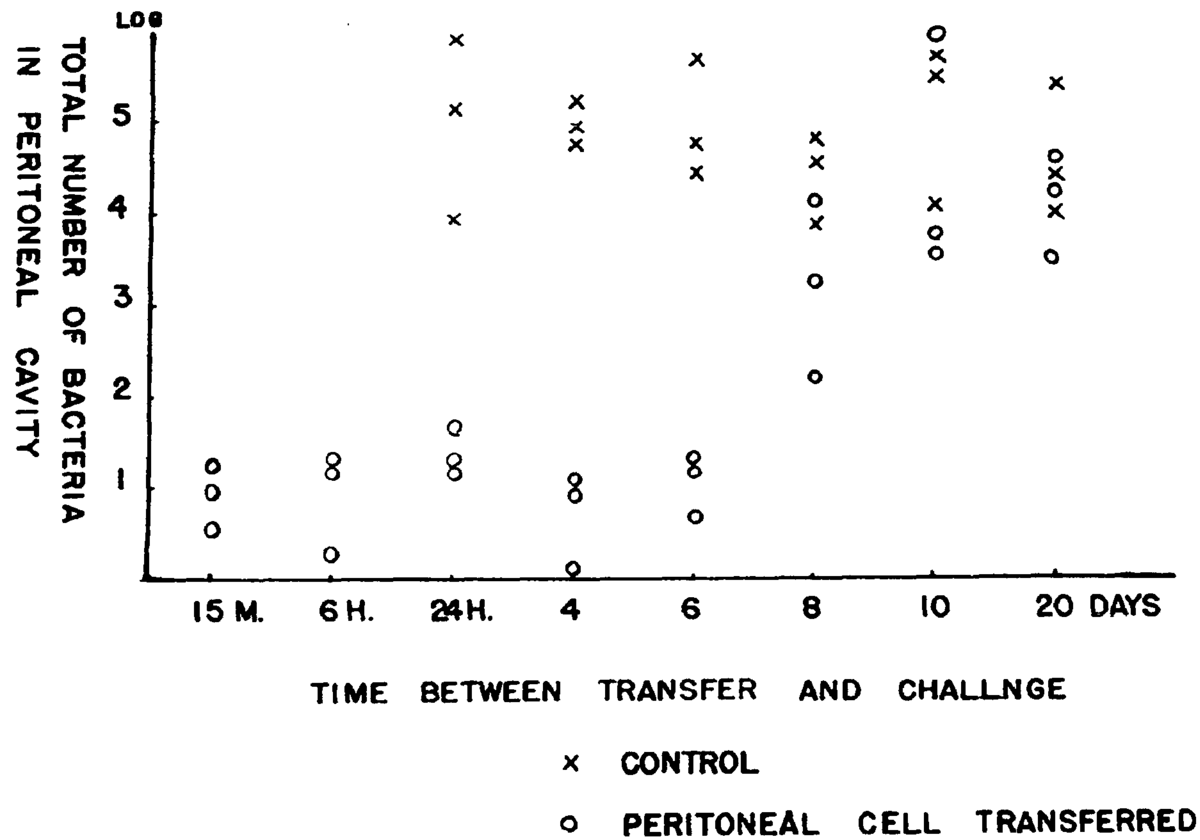

FIG. 8. INFLUENCE OF TIME FACTOR TO CLEARANCE EFFECT
(dd/K▲ STRAIN ADUL MOUSE)